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Pressurized Liquid Extraction of Soil Microbial Phospholipid and Neutral Lipid Fatty Acids

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Soil microbial lipid biomarkers are indicators of viable microbial biomass and community structure. Pressurized liquid extraction (PLE) of soil phospholipid fatty acids (PLFA) and neutral lipid fatty acids (NLFA) was compared to a conventional extraction method in four soils with differing physical and chemical properties. PLE efficiency was greater than that of the conventional method for about half of the saturated PLFA and for selected other Gram-positive (i16:0) and Gram-negative bacteria (18:1ω7c) PLFA, fungal PLFA (18:2ω6,9c), and eukaryotic NLFA from a coarse-textured soil. Lipids extracted by the two methods did not indicate a significant difference in microbial community structure data. Principle component analysis revealed that PLFA clustered by location, with data indicating that the group of microbes contributing the greatest weight differed among soils. Overall, the PLE method proved to be more efficient at extracting soilborne microbial lipids while not altering microbial community information. These advantages indicate the PLE method is robust and well-suited to soil microbial ecology research.

KEYWORDS: Soil microbial lipid analysis; pressurized liquid extraction; phospholipid and neutral lipid fatty acids; fatty acid methyl esters

INTRODUCTION

Pressurized liquid extraction (PLE)—also known as accelerated solvent extraction (ASE) or pressurized hot solvent extraction (PHSE)—is widely used to extract organic compounds from a variety of food, environmental, and biologic samples (*1*). Target analytes include persistent organic contaminants [polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins], pesticides, metals (arsenic, mercury), drug residues (antibacterials, steroids), food toxins, and food and environmental lipids (*1*−*8*). Commonly reported advantages of PLE over traditional extraction techniques such as Soxhlet extraction include improved extraction efficiency, more rapid sample processing times, and less reagent use.

Four parameters affecting PLE efficiency include temperature, pressure, solvent type, and extraction time (*5*). High pressures (>10 MPa) serve to keep solvents from boiling and can increase extraction of PAHs (*2*). High temperatures increase chemical solubility and diffusion rates and reduce surface tension, allowing enhanced penetration of solvents into matrix pores (*5*). Increasing the extraction temperature from 50 to 100 °C increased PAH, PCB, and organochlorine pesticide recovery using PLE,; however, using temperatures >100 °C did not increase extraction of organochlorine pesticides from aged, contaminated soil (*9*, *10*). Extraction times can be short (4−5 min) and include multiple cycles. A variety of solvents and solvent mixtures can be used.

Success with PLE of hydrophobic PAH and organochlorine pesticides and related compounds indicates that PLE may effectively extract soil lipids. Lipids present in soil can be of microbial or plant origin, possess different chemical properties, and can be located in membranes or external structures. Phospholipid fatty acids (PLFA) present in viable prokaryotic and eukaryotic microorganisms serve in membrane fluidity and turn over rapidly upon cell death (*11*, *12*). Neutral lipid fatty acids (NLFA) serve as energy storage structures for eukaryotic organisms including soil fungi (*13*). Their analysis can identify treatment differences between environmental variables including soil cover, land management, contaminants present, and fire effects (*14*−*16*). The use of PLE to extract soil microbial lipids may enhance the capability to detect differences due to increased extraction efficiency. However, different solvent systems can be more or less effective due to differences in lipid polarity and location (membrane bound vs external storage vesicles).

The most widely used extraction solution for soil microbial lipids, which was first described for fish tissue, is the Bligh and Dyer three-component mixture of methanol, chloroform, and water at a 2:1:0.8 ratio, followed by chloroform-only extraction (*17*). A modification of the original Bligh and Dyer method is to use 50 mM phosphate buffer in place of water for the extraction of microbial lipids from sediment (*18*). The buffer enhances methanol and chloroform contact with microbial cells, and the combination of organic solvents accommodates a range in lipid polarity. The three-component single-phase split is accomplished by adjusting the chloroform and aqueous phases to achieve a final methanol/chloroform/aqueous ratio of 1:1:0.9. The procedure was expanded to include derivitization procedures for the lipid extract to recover fatty acid methyl esters using a mild alkaline hydrolysis in methanol (*18*). This modified Bligh and Dyer

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Table 1. Selected Soil Properties

				Mehlich 1 extractable (mg kg^{-1})				0.1 M KCl extractable			
soil location	management	series	pH (1:1 soil/water)	P	K	Ca	Na	NH_4−N	NO_3−N	SOC (g kg^{-1})	total N (g kg^{-1})
Georgia	annually tilled peanut−cotton	Tifton fine loamy sand	6.7	55	112	977	33	8.8	10.4	7.41	0.49
Kansas	undisturbed tallgrass prairie	Ivan silty clay loam	8.1	20	341	1223	36	0.1	3.1	32.2	2.8
Indiana	no-till (7 years) corn−soybean	Chalmers silty clay loam	5.1	31	85	587	30	8.4	23	28.3	2.5
Pennsylvania	no-till (8 years) corn−soybean−wheat	Hagerstown silt loam	6.2	8.7	70	1062	30	14.2	4.4	19.3	1.9

method has been demonstrated to effectively extract microbial lipids from aquifers (*19*), sediment (*18*), roots (*12*, *20*), and soils under different management and climate (*14*, *15*, *21*−*27*). Physical disruption was shown to enhance eukaryotic PLFA yield in prairie soil (*12*), and it is possible that physical disruption, in addition to high pressure and temperature, could lead to even greater extraction efficiency. Other researchers (*3*) found PLE was effective at extracting PLFA from a variety of environmental samples, including one soil. Their work indicated that PLE resulted in substantially higher eukaryotic PLFA, but did not address NLFA. Pressurized liquid extraction was also found to extract total lipid fatty acids from different soils with the Bligh and Dyer solution or chloroform/methanol; however, separation and quantification of PLFA and NLFA were not attempted (*4*). The objective of this study is to use PLE both to extract total lipids from different soils and to evaluate microbial community structure using both PLFA and NLFA. Studies were conducted using soils with varied texture and chemical properties.

MATERIALS AND METHODS

Sample Processing and Experiment Layout. Soil samples were collected from an annually tilled cotton (*Gossypium hirsutum*)−peanut (*Arachis hypogaea*) rotation field near Tifton, GA, an undisturbed tallgrass prairie from the Konza Prairie Biological Station near Manhattan, KS, a no-till corn (*Zea mays*)−soybean (*Glycine max*) rotation field near West Lafayette, IN, and a no-till corn−soybean−wheat (*Triticum aestivum*) rotation field near State College, PA. The soils sampled are classified as fine-loamy, kaolinitic, thermic Plinthic Kandiudults (Tifton loamy sand, GA); fine-silty, mixed, superactive, mesic Cumulic Hapludolls (Ivan silty clay loam, KS); fine, mixed, semiactive, mesic Typic Hapludalfs (Hagerstown silt loam, PA); and fine-silty, mixed, superactive, mesic Typic Endoaquolls (Chalmers silty clay loam, IN). Samples were collected from the upper 20 cm, sieved through a size no. 6 sieve (3.35 mm diameter openings), frozen at −20 °C, lyophilized, and ground with a mortar and pestle. Selected soil properties are summarized in **Table 1**. Two experiments were conducted. The first experiment tested the effects of temperature (ambient and 100 °C), extraction solution (2:1 v/v methanol/chloroform or modified Bligh and Dyer solution), and pressure (ambient and 11 MPa) on PLFA extraction from the Tifton fine loamy sand. The methanol/chloroform solvent mixture was tested to evaluate the effects of the aqueous buffer, to determine if omitting the buffer would reduce extraction efficiency. If not, the 2:1 methanol/chloroform extract could be dried and directly applied to silica gel cartridge (below) without the overnight separation step. The second experiment evaluated three different soils ranging in texture and chemical composition with the two most efficient extraction methods from experiment 1 (**Table 2**).

Extraction Protocols (A and B). *A. Modified Bligh and Dyer Extraction.* Ten grams of dry soil was extracted with 100 mL of modified Bligh and Dyer solution—2:1:0.8 methanol/chloroform/disodium citrate buffer (0.2 M, pH 4)—in a 250 mL square glass bottle. The bottles were agitated on a rotary shaker for 2 h at 220 rpm and placed in a freezer (−20 °C) overnight for soil to settle and permit pipetting of supernatant the following day. Seventy-five milliliters of the supernatant was transferred to a 250 mL separatory funnel, fortified with 20 mL each of disodium citrate buffer and chloroform to form a 1:1:0.9 ratio of methanol/chloroform/citrate buffer, shaken, and separated overnight. The lower layer was drained and evaporated at 50 °C under a directed stream of N_2 gas. Note that this protocol was completed in 3 days.

Table 2. Experimental Protocols for the Study

soil	treatment	temp	pressure	agitation	solvent
		Experiment 1			
Georgia	conventional	ambient	ambient	rotary shaker, 220 rpm, 2 h	BD
Georgia	PLE-MC-amb	ambient	11 MPa	none	2:1 M:C
Georgia	PLE-MC-100	100 °C	11 MPa	none	2:1 M:C
Georgia	PLE-BD-amb	ambient	11 MPa	none	BD
Georgia	PLE-BD-100	100 °C	11 MPa	none	BD
		Experiment 2			
Kansas	conventional	ambient	ambient	rotary shaker, 220 rpm, 2 h	BD
Kansas	PLE-BD-100	100 °C	11 MPa	none	BD
Indiana	conventional	ambient	ambient	rotary shaker, 220 rpm, 2 h	BD
Indiana	PLE-BD-100	100 °C	11 MPa	none	BD
Pennsylvania	conventional	ambient	ambient	rotary shaker, 220 rpm, 2 h	BD
Pennsylvania	PLE-BD-100	100 °C	11 MPa	none	BD

B. PLE Extraction Protocols. A five-channel pressurized liquid extractor (Fluid Management Systems, Inc., Waltham, MA) was used for the experiment. Ten gram lyophilized soil samples were mixed with 10 g of sand (50−250 μm diameter—cleaned by baking at 500 °C for 5 h in a muffle furnace) and loaded into 25 × 125 mm stainless steel tubes between two 20 g layers of sand. The tubes were extracted with the modified Bligh and Dyer solution using the following temperature and pressure program: tubes were filled (1 min), pressurized to 11 ± 0.7 mPa and simultaneously heated to 100 ± 8 °C (hold 15 min), cooled to < 30 °C (10 min elapsed), and depressurized (0.1 min). Ambient temperature samples were not heated. The sequence was repeated three times and followed by removal of all fluids from the PLE instrument using pressurized N_2 gas. Seventy-five milliliters of the extract was transferred to a 250 mL separatory funnel, fortified with 20 mL each of disodium citrate buffer and chloroform, shaken, and separated overnight. The total extract volume not used was recorded for later corrections. The lower layer was drained and evaporated at 50 °C under N_2 gas. The protocol was completed in 2 days.

Ten grams of lyophilized soil was also extracted with PLE at ambient or elevated temperature similar to above but substituting the 2:1 methanol/chloroform solution in place of the modified Bligh and Dyer solution. A 75 mL portion of the extract was transferred directly to a centrifuge tube and dried at 50 °C under N_2. The total extract volume not used was recorded for later corrections. The protocol was completed in 1 day.

Lipid Separation, FAME Derivatization, and Analyses (A and B). Dried lipid extracts were transferred to a silica gel cartridge (Thermo Scientific, 500 mg silica gel, 6 mL volume) with 4 × 0.5 mL of chloroform. Neutral lipids, glycolipids, and phospholipids were eluted from the column with an additional 8 mL of chloroform, followed by 10 mL of acetone and 10 mL of methanol, respectively. Lipid separation efficiency for phospholipids and neutral lipids was tested using L-α-phosphatidylcholine (Sigma P4139) and tristearin (Acros 422270250) and found to be 96 and 105%, respectively. Samples were saponified and methylated by dissolving the dried neutral lipid and phospholipid extracts in 0.5 mL of chloroform and methanol and 1 mL of 0.2 M methanolic KOH and placing tubes in a 60 °C water bath for 1 h. After cooling, 2 mL of deionized water was added, and fatty acid methyl esters (FAME) were extracted two times using 2 mL of hexane. The combined extracts were dried at 40 °C under N_2, suspended in 1.0 g of hexane containing 20 ng μL^{-1} of the internal standard methyl nonadecanoate (Matreya 1029), fortified with 5 ng μL^{-1} of the internal standard 2-chlorolepidine, and transferred to GC vials.

Both PLFA and NLFA fractions were analyzed using an Agilent 6890 GC with a DB5-MS column (30 m × 250 μm i.d. × 0.25 μm film thickness; Agilent 122-5532) and a flame ionization detector (FID). Helium was the carrier gas (1.0 mL min^{-1} constant flow), and the temperature program was as follows: from 50 to 170 °C at 20 °C min^{-1}; from 170 to 270 °C at 5 °C min^{-1}. The injector and detector temperatures were 220 and 300 °C, respectively. Hydrogen and air flow rates into the detector were 30 and 350 mL min^{-1}, respectively. Bacterial acid methyl esters mix (BAME; Matreya 1114), methyl 10(*Z*)-heptadecenoate (Matreya 1203), and methyl 10-methylhexadecanoate (Matreya 1792) were used to identify peaks, and the internal standard methyl nonadecanoate was used to quantify data.

Peak assignments for PLFA and NLFA were confirmed using a Thermo Scientific Trace GC-DSQ II mass spectrometer (San Jose, CA). Column, carrier gas, injector temperature, and oven temperature program were the same as for the GC-FID analysis. Analyses were conducted in the electron impact (70 eV) and positive chemical ionization (PCI) modes (methane as the reagent gas at 2.5 mL min^{-1}), and mass spectrometer scanning m/z^+ was from 200 to 400. In PCI mode the electron energy and current were set to 120 eV and 50 μA, respectively. Each of the methyl esters produced ions corresponding to $M - 1$, $M + 1$, $M + 29$, and $M + 41$, which are useful for molecular weight determination. Tentative assignments of methyl ester peaks not present in the BAME mix were made by mass spectral interpretation and comparison to the NIST Mass Spectral Database version 2.0 (NIST, Gaithersburg, MD).

Twenty-one FAME were identified in the PLFA fraction and were grouped into saturated, Gram-positive and Gram-negative bacteria, and fungi according to the guidelines of McKinley et al. (*26*): (a) saturated—12:0, 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0; (b) Gram-positive bacteria—i15:0, a15:0, i16:0, i17:0, a17:0, and 10me18:0; (c) Gram-negative bacteria—2-OH 14:0, 16:1ω7c, cy17:0, 18:1ω7c, and cy19:0; and (d) fungi—16:1ω5c, 18:1ω9c, and 18:2ω6,9c. Six FAME from the eukaryotic neutral lipid fraction were chosen to represent fungal biomass and included 16:0, 16:1ω5c, 18:0, 18:1ω9c, 18:2ω6,9c, and 20:0. Nomenclature is as follows: carbon chain length:number of double bonds, location of double bonds from CH_4 end of molecule, cis configuration. Prefixes i, a, OH, me, and cy indicate iso or anteiso branching and hydroxy, methyl, or cyclopropyl groups, respectively.

Statistics. The Georgia soil was replicated five times and all others three times for each extraction procedure. For experiment 1, FAME data from the only Georgia soil were subjected to an ANOVA using the Proc MIXED procedure, and for experiment 2, FAME data from the conventional and PLE-BD-100 for all four soils were subjected to an ANOVA using the Proc MIXED procedure in SAS v. 9 (Cary, NC). All means were separated using a paired *t* test with a significance level of 0.05. Principal component analysis was conducted using Proc PRIN procedure in SAS v. 9, and eigenvalues >1 were subjected to an ANOVA using Proc MIXED.

RESULTS

Experiment 1. Nineteen FAME were identified in the PLFA fraction of the Georgia soil sample extracted conventionally or using PLE with the modified Bligh and Dyer solution (**Figure 1**). The membrane-bound PLFA in the Gram-positive (i15:0, a15:0, i16:0, a17:0, i17:0, and 10me18:0) and Gram-negative bacteria (16:1ω7c, cy17:0, 18:1ω7c, and cy19:0) and fungi (16:1ω5c, 18:2ω6,9c, and 18:1ω9c) were extracted with greater efficiency using the modified Bligh and Dyer solution than the methanol/chloroform mixture (**Figure 2**). Levels of PLFA 14:0, i15:0, a15:0, 15:0, i16:0, 16:1ω7c, 16:1ω5c, a17:0, i17:0, cy17:0, 17:0, 18:0, 10me18:0, and 20:0 were similar for the modified Bligh and Dyer solution either by conventional shaking or PLE and significantly lower or absent for protocols using the methanol/chloroform solution (**Figure 2**). Soil extracted using the modified Bligh and Dyer solution and PLE at 100 °C increased PLFA 16:0, 18:2ω6,9c, 18:1ω9c, 18:1ω7c, cy19:0, and 20:0 yield, as compared

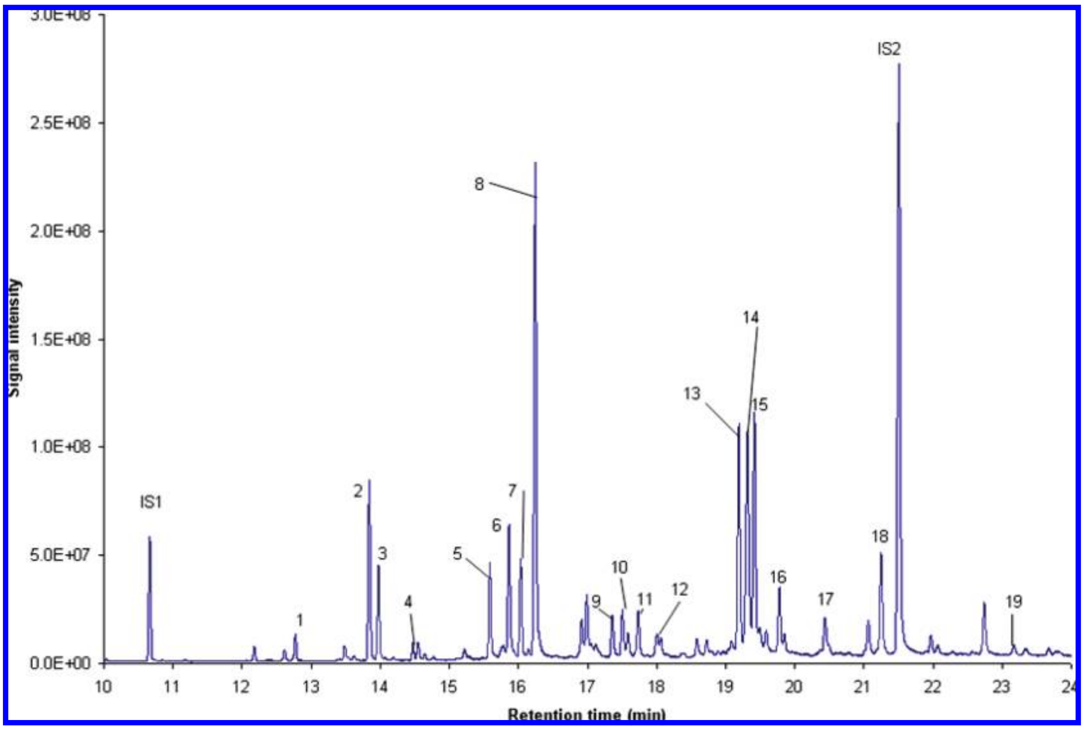

Figure 1. Typical gas chromatogram obtained from analysis of FAME extracted from Georgia soil. Numbers near peaks are as follows: (IS1) internal standard 2-chlorolepedine; (1) 14:0; (2) i15:0; (3) a15:0; (4) 15:0; (5) i16:0; (6) 16:1ω7c; (7) 16:1ω5c; (8) 16:0; (9) i17:0; (10) a17:0; (11) cy17:0; (12) 17:0; (13) 18:2ω6,9c; (14) 18:1ω9c; (15) 18:1ω7c; (16) 18:0; (17) 10me18:0; (18) cy19:0; (IS2) internal standard 19:0; (19) 20:0. Chromatograms for modified Bligh and Dyer extraction (conventional), PLE + Bligh and Dyer at ambient temperature, and PLE + Bligh and Dyer at 100 °C were all similar to the figure.

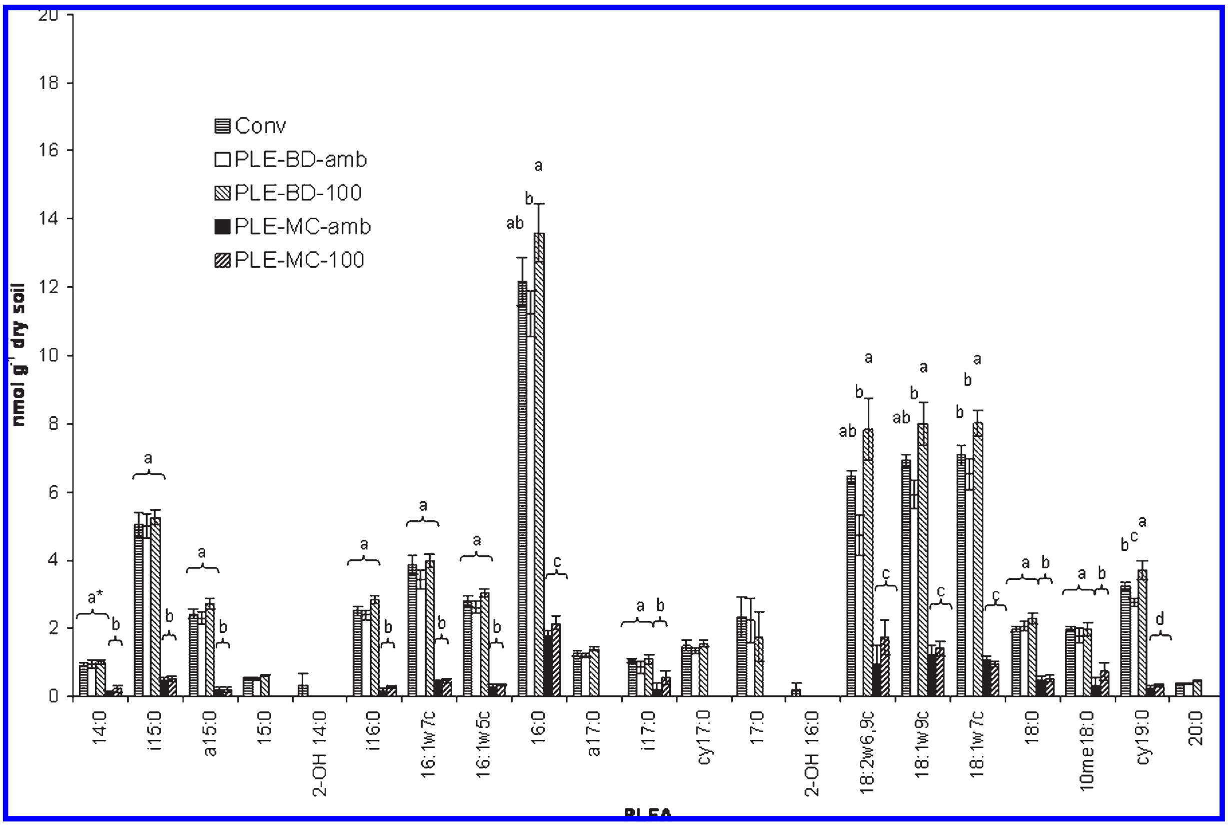

Figure 2. Microbial PLFA biomarkers extracted from Georgia soil using either conventional or PLE methods. Means with the same letter for a particular PLFA are not different at the $p < 0.05$ significance level. PLFA without statistical letters are discussed in the text. Lines are ±1 standard error of the mean. PLE, pressurized liquid extraction; BD, modified Bligh and Dyer solution; MC, methanol–chloroform solution; amb, ambient temperature; 100, 100 °C temperature.

to the PLE at ambient temperature. Levels of 18:1ω7c and cy19:0 were significantly greater for PLE when using the modified Bligh and Dyer solution at 100 °C than for any other protocol or solvent used. Our results did not show that mono- and polyunsaturated fatty acids were sensitive to the temperatures evaluated. For experiment 2, the conventional method (ambient temperature, ambient pressure, modified Bligh and Dyer solution, rotary shaker—220 rpm for 2 h) and a PLE method (100 °C, 11 MPa, modified Bligh and Dyer solution, 3 × 15 min extraction) were chosen to evaluate the effectiveness of PLE in the different soil types.

Experiment 2. Total levels of PLFA extracted from the Georgia, Indiana, Kansas, and Pennsylvania soils using the conventional and PLE (100 °C, 11 MPa, modified Bligh and Dyer solution, 3 × 15 min extraction) methods were 68 and 75, 62 and 71, 131 and 137, and 69 and 74 nmol g^{-1} of dry soil, respectively, and means for each soil were not significantly different between extraction methods at each site. The PLE method did extract significantly higher amounts of certain PLFA, especially saturated fatty acids, as compared to the conventional method (**Table 3**). Microbial group-specific PLFA differences between methods included i16:0, a Gram-positive bacterial PLFA, in the Pennsylvania soil, 10me18:0, a Gram-positive bacterial PLFA, in the Kansas soil, 18:2ω6,9c, a fungal PLFA, in the Indiana soil, and 18:1ω7c, a Gram-negative bacterial PLFA, in the Georgia soil (**Table 3**). All except the 10me18:0 were extracted with greater efficiency with the PLE method. Other PLFA extracted from the Indiana, Kansas, and Pennsylvania soil samples were similar between the conventional and PLE methods.

Total NLFA extracted were 37 and 47, 103 and 110, 31 and 39, and 39 and 37 nmol g^{-1} of dry soil using the conventional and PLE methodologies for Georgia, Kansas, Indiana, and Pennsylvania soils, respectively. Means of NLFA amounts extracted using PLE were significantly greater for Georgia and Indiana soils, but Kansas and Pennsylvania soils were not different between methods. Individual levels of NLFA varied by site, and PLE extracted greater amounts of 16:1ω5c, 16:0, 18:1ω9c, and 18:0 in the Georgia soil and greater amounts of 16:1ω5c, 16:0, and 18:0 in the Indiana soil (**Table 3**). Either method extracted similar levels of NLFA in Kansas and Pennsylvania soils.

PLFA data were converted to a mole fraction basis to determine if the extraction method altered the microbial community structure information obtained. Biomarkers for fungi, Gram-negative bacteria, and Gram-positive bacteria were summed, and each group was reported as a fraction of the total. The PLE methodology resulted in higher mole fractions of Gram-positive bacteria from the Georgia soil and fungi from the Indiana and Kansas soils; however, the differences were minor (**Table 3**). For the finer-textured soils (Indiana, Kansas, and Pennsylvania) fungi represented about 20% of the mole percent, whereas the value was about 35% in the coarser-textured Georgia soil. Gram-positive and Gram-negative bacterial PLFA accounted for about 40% each in the Kansas, Indiana, and Pennsylvania soils and for 30–35% in the Georgia soil.

Principal components (PC) 1 and 2 explained 45 and 23% of the variability, with eigenvalues of 9.4 and 4.9, respectively. The extraction method had less effect on PC1 or PC2, as compared to location (**Figure 3a**). Eigenvectors plotted for PC1 showed that fungal derived PLFA (18:2ω6,9c, 18:1ω9c, 16:1ω5c) weighted PC1 positively, whereas Gram-negative bacterial PLFA (cy17:0, cy19:0) and Gram-positive bacterial PLFA (i15:0, a15:0, i16:0, a17:0, 10me18:0) weighted PC1 negatively (**Figure 3a**). Georgia soil had the highest values for PC1, whereas Kansas soil exhibited

Table 3. Microbial Lipids Extracted from Different Soils Using either the Conventional or PLE (100 °C, Bligh and Dyer Extraction Solution) Method[a]

	Georgia		Indiana		Kansas		Pennsylvania	
	conv	PLE	conv	PLE	conv	PLE	conv	PLE
	nmol g^{-1} dry of soil							
PLFA								
saturated								
14:0	0.92	1.01	0.64	0.79	0.98	1.02	0.76	0.82
15:0	0.53	0.61	0.38	0.44	0.76*	0.85*	0.40*	0.47*
16:0	12.2*	13.6*	6.85*	8.93*	12.8*	14.7*	7.39*	9.40*
17:0	2.30	1.75	1.80	2.09	2.43	5.33	1.75	1.83
18:0	1.96	2.29	1.56*	2.06*	3.22*	3.55*	1.62	1.85
20:0	0.39	0.46	0.32*	0.46*	0.62*	0.82*	0.32	0.42
Gram-positive								
i15:0	5.04	5.25	6.08	6.52	9.97	10.1	6.45	6.88
a15:0	2.42	2.71	4.32	4.65	11.6	10.9	4.88	5.16
i16:0	2.52	2.84	2.34	2.68	6.59	7.01	2.55*	3.00*
a17:0	1.27	1.40	1.48	1.64	5.25	5.45	1.35	1.52
i17:0	1.05	1.10	0.55	0.71	0.95	0.85	0.78	0.55
10me18:0	1.99	1.98	1.69	1.88	4.40*	4.22*	2.64	2.41
Gram-negative								
2-OH 14:0	0.34	nd	nd	nd	0.31	0.11	nd	nd
16:1w7c	3.86	3.97	4.79	5.16	5.76	5.41	4.99	5.21
18:1w7c	7.08*	8.02*	5.76	6.34	10.4	10.5	8.80	9.16
cy17:0	1.49	1.56	2.06	2.33	2.91	2.92	1.90	2.12
cy19:0	3.24	3.71	5.46	5.99	19.0	19.6	5.95	5.80
fungi								
16:1w5c	2.80	3.04	2.81	3.16	4.36	4.50	2.79	3.00
18:2w6,9c	6.44	7.83	1.46*	2.06*	3.79	4.21	1.75	1.96
18:1w9c	6.91	7.99	5.29	5.92	10.8	11.3	5.30	5.06
NLFA								
16:1w5c	7.20*	8.43*	7.82*	8.84*	16.8	18.2	11.6	11.0
16:0	10.8*	13.5*	6.20*	8.60*	24.1	27.1	8.00	7.93
18:2w6,9c	5.85	6.35	2.73	3.60	8.84	9.00	4.12	3.30
18:1w9c	8.06*	12.0*	4.45	6.25	41.9	42.5	5.26	5.05
18:0	1.48*	2.61*	1.44*	2.29*	2.87	3.82	1.77	1.96
20:0	3.52	3.62	8.12	9.22	8.59	9.40	8.14	7.95
PLFA	mole fraction							
Gm+	0.31*	0.30*	0.37	0.37	0.40	0.40	0.37	0.38
Gm−	0.35	0.34	0.41	0.40	0.40	0.40	0.43	0.43
fungi	0.35	0.37	0.22*	0.23*	0.20*	0.21*	0.20	0.19

[a] Means between methods for a particular soil within a category (PLFA, NLFA, PLFA mole fraction) followed by an asterisk are significantly different ($p < 0.05$). nd, not detected.

the lowest (**Figure 3a**). Eigenvectors plotted for PC2 indicate that several Gram-negative and Gram-positive bacterial PLFA (i15:0, cy17:0, 16:1ω7c, 18:1ω7c) weighted PC2 positively, whereas other Gram-positive (i16:0 and a17:0) and Gram-negative (cy19:0) bacterial and fungal (18:2ω6,9c, 18:1ω9c) PLFA weighted PC2 negatively (**Figure 3b**).

DISCUSSION

Membrane-bound PLFA were extracted more efficiently from the Georgia sample using the modified Bligh and Dyer solution rather than the methanol/chloroform mixture. The modified Bligh and Dyer solution (2:1:0.8 methanol/chloroform, 50 mM phosphate buffer) was also more efficient at extracting PLFA than a 2:1 methanol/chloroform mixture from pure microbial biomass samples of *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium fortuitum*, *Bacillus subtilus*, *Saccharomyces cerevisiae*, and *Aspergillus niger* (*3*). Our experiments showed that lipid extraction from the Georgia soil sample with PLE was more efficient than a conventional method using a rotary shaker and that increasing the PLE temperature to 100 °C improved results obtained with PLE. This was the case with the fungal PLFA 18:2ω6,9c and 18:1ω9c, possibly indicating that cell walls of fungi are more resistant to solvents than bacterial cell membranes (**Figure 2**) and that higher temperature could overcome this limitation. The increased temperature had no apparent negative impacts on the mono- and polyunsaturated fatty acid content.

Higher amounts of saturated PLFA (15:0, 16:0, 18:0, and 20:0) were extracted from soil samples using PLE, indicating this to be a more robust extraction method (**Table 3**). Although no inferences into soil microbial community structure can be made from these saturated PLFA, they are useful for estimating total viable microbial biomass (*26*). Additionally, the PLE procedure could lead to higher amounts of extractable lipid phosphate (*18*). The increased NLFA extracted from the Georgia sample using PLE could be related to that soil's low clay and SOC content (**Table 1**), both of which can reduce the extraction efficiency of organic chemicals (*27*). Corn residue was present in the Indiana soil sample, and one possible explanation for increased NLFA found is that the PLE conditions improved extraction of fungal NLFA from colonized residue not removed by the original soil processing. Regardless of the explanation, the PLE proved a more robust protocol for soil microbial NLFA in certain soils (**Table 3**). The high-pressure and temperature PLE extracted similar amounts of NLFA, as compared to the conventional rotary shaker method, from the Kansas and Pennsylvania soil samples (**Table 3**).

Interpretations of microbial community structure were similar regardless of extraction method (**Table 3**). The significant differences were small and reflect the low variance associated with means generated from the conversion of data from nanomoles per gram of dry soil to mole fraction. Principal component analysis indicated that location and inherent soil chemical and physical properties more strongly clustered PLFA than the choice of method used (**Figure 3**). The analysis also indicated that fungal PLFA were more important indicators of variability in the Georgia sample, as opposed to bacteria in other samples. High-pressure and -temperature characteristics of PLE appeared to make the extraction more efficient in the coarse-textured soil, but not in the finer-textured soils. Increased PLFA were found in soil collected from the Konza Prairie Biological Station, as compared to a coarser-textured short grass steppe, but a lower mole fraction of fungal PLFA, as compared to a coarser-textured soil (*28*). This could indicate room for improvement in regard to extraction efficiency of fungal PLFA in general, as more PLFA were extracted from the Kansas and Pennsylvania soil samples, but they represented a lower mole fraction (**Table 3**).

In conclusion, the PLE extracted greater amounts of PLFA and NLFA from certain soil samples with different physical and chemical properties. If available in a laboratory, the use of PLE is a valid and robust means of extracting soil microbial lipids. Additionally, several PLFA were extracted at greater efficiency when the PLE was operated at 100 °C as opposed to ambient temperature. However, using the PLE did not result in greatly different mole fraction information, as compared to a conventional method, when used in conjunction with the modified Bligh and Dyer solution. Principal component analysis also indicated that method choice had little effect on microbial structure information. This is advantageous in regard to comparisons of data sets extracted using different methods. Finally, simultaneous extraction of other molecules of interest (e.g., pesticides, PAHs) may be possible and lead to faster experimental protocols.

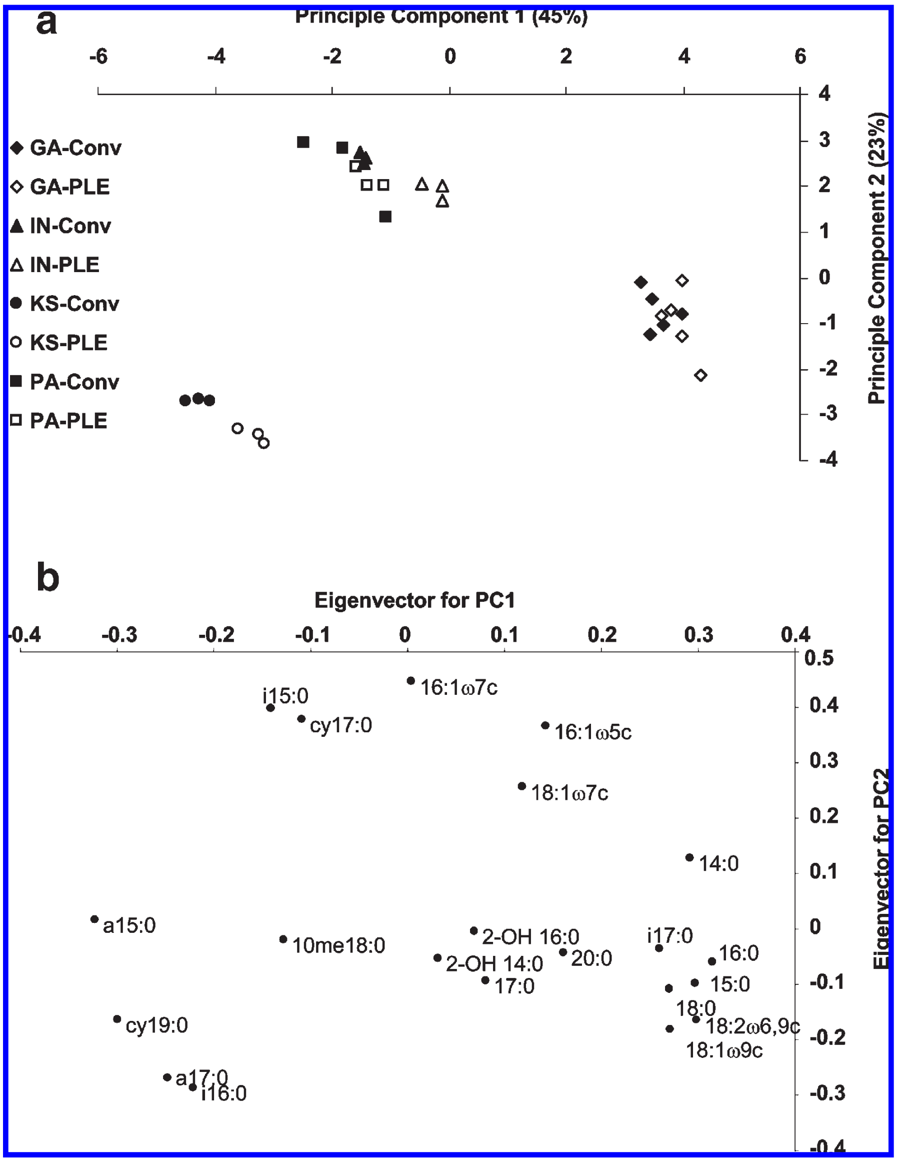

Figure 3. (**a**) Scatterplot of PC1 and PC2 obtained by analyzing the mole fraction of all PLFA extracted from soil. Both PCs were significantly different from 0 at the $p < 0.05$ level. (**b**) Scatterplot of eigenvectors contributing to PC1 and PC2. Labels next to points indicate PLFA.

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